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Characteristics of Limbic Seizures Evoked by Peripheral Injections of Lithium and Pilocarpine

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PERSINGER, M. A., K. MAKAREC AND J.-C. BRADLEY. *Characteristics of limbic seizures evoked by peripheral injections of lithium and pilocarpine.* PHYSIOL BEHAV 44(1) 27–37, 1988.—The characteristics and consequences of limbic seizures evoked by single peripheral injections of lithium (3 mEq/kg) and pilocarpine (30 mg/kg) were investigated over a three-year period. The seizures occurred when 3 mEq/kg of lithium was followed 4 to 28 hours later by 30 mg/kg of pilocarpine. The seizures did not occur if the intervals were shorter or longer or if the pilocarpine preceded the lithium. The acute mortality or persistent aphagia and adipsia could be compensated by postseizure injections of aceproмazine and a special milk-bread diet. Gender, age and preinjection environmental effects, but not hybrid genetics, influenced the seizure onset latency. Fifty to 100 days after the seizures massive lesions were found in the entorhinal-pyriform cortices, amygdala and selected thalamic groups.

Lithium Pilocarpine Amygdala Seizure Pyriform cortex Thalamus Hybrid
Sex differences Limbic seizures Hippocampus Age Aphagia/adipsia

LIMBIC seizures that are associated with sniffing, mouth automatisms, stereotyped forelimb movements and dorsoflexion of the head during a full upright stance have been precipitated by both intracerebral and peripheral injections of cholinergic compounds (6, 10, 11, 14); both the pattern of the seizures and the ensuing brain damage are similar to that produced by kainic acid (6). Usually relatively large dosages of a peripherally injected cholinergic compounds are required to evoke limbic seizures. For example, Turski *et al.* (14) noted that limbic seizures (with behavior and electrical concomitants) and severe brain damage required 400 mg/kg of pilocarpine.

However Honchar, Olney and Sherman (6) have reported a model that produced similar obvious (within 24 hours) brain damage following limbic motor seizures that are evoked by only 30 mg/kg of subcutaneously-injected pilocarpine if lithium (3 mEq/kg) was given in a similar manner 24 hours previously. One of their biochemical tags of the brain-related consequences was a metabolite (d-*myo*-inositol-1-phosphate) of the phosphoinositides, that are assumed to be involved with receptor-mediated processes in the brain. Whereas this metabolite increased by only a factor of 2 or 4 when just lithium or pilocarpine in the above dosages were given singly, the metabolite increased by a factor of 40 when the lithium was followed 24 hours later by pilocarpine.

We considered the implications of this model to have great theoretical and applied significance in light of 1) the role of cholinergic mechanisms (6) in the maintenance of glutamate and aspartate within intrasynaptic space and 2) the frequent use of lithium, often with a cholinergic-affecting neuroleptic, in clinical (particularly geriatric) settings. In addition, since the pattern of brain damage is similar to what has been reported in temporal lobe epileptic patients (4), this model might be useful for delineating and understanding the development of behavioral changes associated with temporal lobe epilepsy. In this article we report the results of the last 3 years of research concerning the characteristics of and conditions that influence lithium-pilocarpine-induced limbic seizures.

SEIZURE CHARACTERISTICS AND CONSEQUENCES

Rats were first injected subcutaneously with 3 mEq/kg of LiCl. After a critical period (Experiment II) they were then given pilocarpine (30 mg/kg; 30 mg/cc saline). The rats demonstrated the expected piloerection, chromodacryorrhea lacrimation and mastication within 5 min; the earliest discernable onset was about 1 min after the injection. Piloerection was more prevalent in younger animals (less than 30 days of age) and older animals (over 100 days). Salivation did not occur as quickly and was more obvious after the seizures had begun.

Between the onset of the pilocarpine effects and the seizure the animals displayed activity levels that ranged from inactive to continuous activity; qualitatively, activity during this period was not an observational predictor of seizure latency or severity. Grooming was conspicuously absent from the animals' behavior patterns during this period; an animal that groomed also did not seizure. The most obvious

behavior during this period involved scratching of the flank with the hindleg. The response could involve either or both legs and was not systematically related to the side of injection. Hindleg scratching existed as a continuum between minimal to almost continuous and was not systematically associated with either seizure latency or with mortality.

The first obvious preseizure behavior involved sniffing that was characterized by rapid movement of the vibrissae and was not associated with exploratory behavior. These events began as early as 5 min after the pilocarpine injection. Initially they lasted for about a sec but increased in both duration and frequency of occurrence; they were associated with staring and blinking and were considered by observers to be one of the best indicators of an impending seizure.

The initial component of the lithium/pilocarpine seizure involved "facial seizures." They were exaggerated sniffing responses that appeared to involve all of the facial musculature and extension of the neck; other parts of the body were not involved. Before the onset the animal was typically stationary. The occurrence ranged from 10 to 15 sec per episode and heralded the onset of more serious seizures.

Two main components could be discriminated from the major seizure pattern: a Phase A component and a Phase B component. The A component (when it occurred 65 to 75% of the time) preceded the Phase B component. Phase A was characterized by extension of the neck followed by retraction of the neck and then a severe backward extension of the head; the latter was often accompanied by flexion of the forelimbs and a tonic dorsal flexion of the tail (scorpion tail). The head and forelimb displacement lasted for approximately 10 to 20 seconds; there was no obvious change in the hindlimbs. In addition, older animals tended to twist the upper portion of their torso to one side. During the Phase A seizure the rat's mouth was typically partially open such that the lower teeth were visible.

Between the termination of the initial Phase A and Phase B, an interlude was apparent. Young rats (60–100 days) generally displayed little more than mouth automatisms and/or facial seizures; they were usually periodic with 20-sec durations. Older animals immediately began to show one of two types of behaviors: "nose dives" or "jumps." The nose dive was defined as moving immediately from a Phase A position and placing the nose in the bedding; in severe cases the back was arched. During this time the forepaws displayed a paddle motion. Jumps were characterized by repeated upward hops (all four limbs off the floor). The back was usually arched and the "scorpion tail" was evident. Piloerection was usually more evident during this time.

Phase B began with enhanced facial seizuring. However, in addition, the rat assumed a fully upright posture for about 20 sec to one minute. From this position the animal often fell backwards. Younger animals landed on their paws (righting reflex responsive) while the older animals tended to fall on their backs or haunches. These types of seizures were displayed for the next few hours; the number of episodes ranged from 2 to 10.

For reference, Phase A and Phase B displays were compared to seizures that are evoked by amygdaloid kindling or systemic injection of kainic acid. The Phase B seizures were perceptually indiscriminable from the typical Stage IV kindled seizure. However the lithium-pilocarpine seizure was more protracted than kindled displays (not surprising in light of the relative brevity of the precipitating stimulus: a short interval of current). Interestingly, both the kindled- and lithium-pilocarpine-seizured males emitted frequent ejaculate (as measured by sperm plugs during the day and days after the seizure onset). On the other hand, systemic kainic acid seizures displayed a slightly longer onset time (30 to 40 min), starting with wet dog shakes and were overtly less severe.

From the time the observation of the seizure was completed until the end of each study each rat was housed singly. During the next 2 to 8 hours the rat constantly displayed some form of seizure activity. The most common forms were: facial display, jumps and wet dog shakes. Oral or perioral movements involving the tongue became obvious. Wheezing sounds (of obvious respiratory origin) and teeth grinding became more prominent. After about 8 hours of this activity the animals became comatose or unresponsive to the environment. Dependent upon age and dosage they usually died during the next 48 hours or entered a period of aphagia and adipsia that could be compensated by a bread and milk diet.

If the animal began to recover and to eat the bread/milk mixture, brief but violent convulsions with durations of approximately one min occurred intermittently for about two weeks. The animal recovered almost immediately from the convulsion and resumed preseizure activities. Seizure forms involved strange (foot paddling) use of forepaws, contortions of the torso (waste-twists), bizarre running movements (similar to picrotoxin-induced seizures) against the cage wall (as if to run up the wall) and boxing positions. Staring spells were frequent. There was a conspicuous lack of grooming behaviors and oily hair for at least a month after the seizures. The rats were hyperreactive to handling and squeaked loudly or bit the experimenter's glove. Sperm plugs (beneath the cages) were an obvious feature of the postseizure period. Pharmacological treatment, as noted below, reduced the seizure-induced mortality but not the pattern of the responses.

EXPERIMENT I: SEIZURE LATENCY AND CHARACTERISTICS

The first study was designed a) to determine the statistical characteristics of the seizure latency and b) to discern if the latency or type of seizure (type B only or A and B) contributed to mortality. (The number of rats that displayed Phase A only seizures was too few to be included in the analysis.)

Subjects

A total of 95 male Wistar albino rats between the ages of 90 and 110 days (obtained from Woodlyn Breeders, Guelph, Ontario or from Charles River in Quebec) were used as subjects (unless otherwise stated this strain and these sources were involved with all consequent experiments).

Procedure

The animals were initially housed (3/cage) within wire cages that were maintained in standard racks. Food and water were available ad lib. The colony room (Paul Field Animal Facility) temperature was controlled by a homeostat and ranged from 68 to 70 degrees F. The light-dark cycle was 12:12 with the light phase beginning at 0800 hours.

Approximately 24 hours before the injection with pilocarpine the animals were removed briefly from their home cages and injected subcutaneously in the flank of the lower right or left leg with 3 mEq/kg LiCl (3 mEq/cc). The next day the animal was injected in the same area with 30 mg/kg of pilocarpine. Each animal was placed in an aquarium and either videotaped or watched by a trained observer. The

time of the first discernable facial seizure and the main seizure [we selected Phase B in order to be compatible with Honchar *et al.* (6)] were recorded to the nearest second. Two to three rats were tested at once with a total of 30 rats tested per month. About half of the rats were tested during the summer of 1985 while the remainder were tested that fall.

Results and Discussion

The means and standard deviations for the latency of onset for the facial seizures and Phase B seizures were 24.1±9.5 and 30.1±9.7 min, respectively. [The correlation (r) between the latency of Phase A and Phase B was .85]. These results indicated a relatively tight dispersion around the seizure onset and are comparable to those reported by Honchar *et al.* (6) who reported a mean of 31.9±3.6 min and by Turski *et al.* (14) who injected 400 mg/kg of pilocarpine (IP) only. To discern if the latency of the facial seizure onset influenced mortality, the rats were divided into four a posteriori groups: 1) death within 6 hours of the seizure, 2) death between 6 and 24 hours after the seizure, 3) death between 24 and 48 hours after the seizure, and 4) rats that were still alive after that period (although they were aphagic and adipsic). The means and standard deviations were 21.2±5.8, 28.0±11.2, 24.0±6.7, and 24.1±14.5 min, respectively. Both analysis of variance, $F(3,90)=1.78$, and Kruskal-Wallis nonparametric analysis [chi-square (3)=2.67,] indicated no significant ($p>0.05$) difference between groups. Thus it appears that latency of seizure onset does not influence mortality. There was no significant difference [chi-square(3)=2.11] between mortality for rats that displayed either a Phase B only or Phase A and B seizure. Although there was no significant difference between the onset of facial seizures, $F(1,92)=3.09$, $p>0.05$, for rats that showed A and B (22.7±9.0 min) or B only (26.7±10.3 min) main seizures, the onset of the phase B seizure was significantly ($p<0.01$) earlier, $F(1,92)=6.45$, for the rats that had B seizures (n=30) only (26.5±10.6 min) compared to the rats that displayed A plus B (n=64) seizures (31.8±8.8 min).

EXPERIMENT II: MANIPULATION OF TEMPORAL FACTORS BETWEEN DRUG INJECTIONS

Although the typical interval between the injection of lithium and the administration of pilocarpine has been 24 hours in order to insure maximum concentrations of Li had occurred within the brain (5), we were interested in the relationship between the time between injections and the seizure latency. In addition, we included a dose-dependent design in this experiment to insure that the optimal concentrations were being used. Because repeated injections of "subthreshold" dosages of lithium and pilocarpine over time might "kindle" the seizure, this question was addressed as well.

Subjects and Method

In part A of this experiment 54 male rats between the ages of 120 and 140 days of age were used as subjects. Rats received either 1.0 mEq/kg, 3.0 mEq/kg or 5.0 mEq/kg of lithium and then either 24 hours or 48 hours later either 10, 30 or 50 mg/kg of pilocarpine. The animals were observed to determine if they either displayed a seizure (Phase B) or did not.

In part B of this experiment 45, 90- to 110-day-old male rats were divided into 9 groups. Eight of the groups were injected with 3 mEq/kg of lithium chloride and then either 0 (same time, but other flank), 0.5, 1, 2, 4, 24, 48 or 72 hr later with 30 mg/kg of pilocarpine. The ninth group was injected with pilocarpine first and then lithium 2 hours later. In part C of the experiment 6 male rats (100 to 110 days of age) were injected with 1.5 mEq of lithium chloride/kg and then 4 hours later with 15 mg/kg of pilocarpine on Mondays, Wednesdays and Fridays for 4 consecutive weeks (12 pairings). After each pairing the animals were observed for one hour.

TABLE 1

NUMBER OF ANIMALS (MAXIMUM 3 PER CELL) THAT SEIZURED AS A FUNCTION OF DOSAGE OF LITHIUM OR PILOCARPINE AND THE LATENCY BETWEEN THE SINGLE SUBCUTANEOUS INJECTION OF LITHIUM AND THE SINGLE SUBCUTANEOUS INJECTION OF PILOCARPINE

	10 mg/kg	30 mg/kg	50 mg/kg
		Pilocarpine (24 hr later)	
LiCl			
1 mEq/kg	0	0	0
3 mEq/kg	1	3	3
5 mEq/kg	3	3	3
		Pilocarpine (48 hr later)	
LiCl			
1 mEq/kg	0	0	0
3 mEq/kg	0	0	1
5 mEq/kg	0	2	3

Results

The results of part A were discrete and clear. As shown in Table 1, although 3 mEq/kg of Li and 30 mg/kg of pilocarpine always generated seizures, larger dosages of Li produced seizures with less pilocarpine. (However these animals usually showed skin lesions of the LiCl injection site.) If the interval between the Li and pilocarpine equaled 48 hours, only the 5 mEq/kg dosage of lithium elicited the seizure.

Part B results support these observations as noted in Fig. 1. The shortest seizure latency occurred for the group that had been injected with 3 mEq/kg of Li and 24 hours later with pilocarpine; the values are comparable to those from Experiment I. However, if the injection intervals were 48 hours or greater no seizures occurred. In addition, if the interval was 2 hours or less, not all animals seizured; the probability of seizuring decreased as the time between the lithium and pilocarpine injections decreased. Although all animals that were injected with lithium and then pilocarpine 2 hours later seizured, none of the animals that were injected in the reverse temporal order seizured.

The results of part C indicated no overt evidence of kindling; despite 12 pairings of 1.5 mEq/kg Li and 15 mg/kg of philocarpine, there was no evidence of Phase A seizures. However "facial seizures" were qualitatively more evident and coprophagia was conspicuous. The former were similar to but not as intense as those associated with the frank seizure. The coprophagia was indiscriminable from that associated (from our experience) with single, higher (e.g., 50 mg/kg) dosages of pilocarpine.

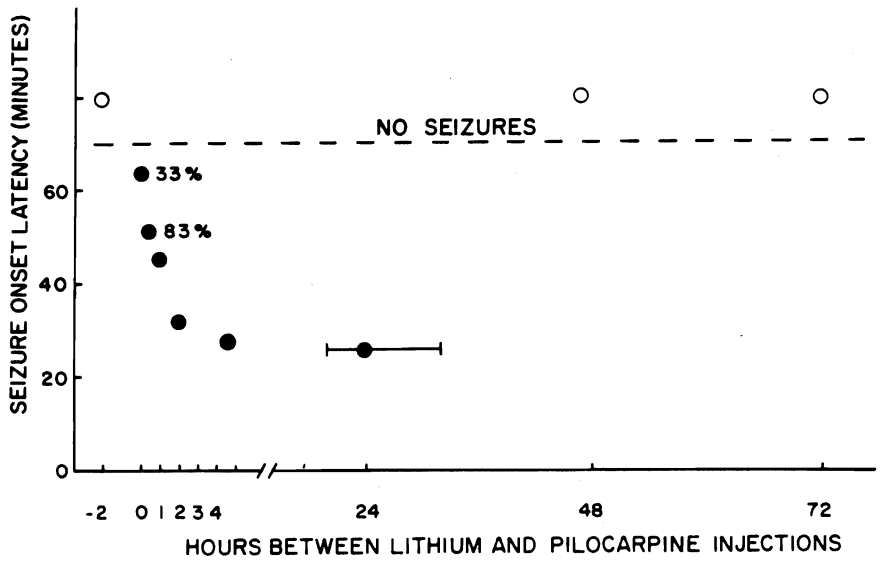

FIG. 1. Onset (in minutes) of the facial component of the limbic seizure as a function of the time between a single subcutaneous injection of lithium chloride (3 mEq/kg) and a single subcutaneous injection of 30 mg/kg of pilocarpine.

EXPERIMENT III: MORTALITY AND PATTERN OF RECOVERY

After 6 to 8 hours after the animal had begun to seizure, it became immobile and then comatose. During the next 24 hours the rat either died or began to slowly recover. The greater the degree of immobility during the first 24 hours after the seizure, the more likely the mortality. However if the animal survived for more than 48 hours, then dietary changes were required to maintain vitality. The animals displayed a syndrome that was very similar to lateral hypothalamic damage; the primary characteristics were aphagia and adipsia. This study was designed to determine the contribution of age to survival and the importance of the novel diet to recovery.

Subjects and Method

A total of 100 male Wistar rats were used in part A of this study. Their ages were 50 days (n=20), 70 days (n=20), 90 days (n=20), 110 days (n=20), and 130 days (n=20). Each animal was injected subcutaneously with 3 mEq/kg of lithium and 30 mg/kg of pilocarpine 24 hours later. Usual injection times were between noon and 1700 hr local time. The animals were checked for vital signs every day for 10 days after the seizure. If the animals survived for at least two days they were given a mixture of diluted condensed (Eagle Brand) milk together with pieces of wheat bread in a small bowl. Because they rarely ate before that time, this meant that the animals had ignored food and water for at least 48 hours. In the second part of this experiment 24 additional 110-day-old male rats that had survived for at least 48 hours following the seizure were divided into two groups: the first group (n=12) was never given the milk and bread mixture and were exposed to Purina rat chow pellets on the floor of the cage and to water ad lib, 2) the second group (n=12) was given the milk and bread mixture; once they had begun to eat this combination (vigorously), rat chow dust was introduced slowly into the mixture. This fading technique was continued until complete dry pellets only were present within the dishes and water was available through usual methods of delivery.

Results

The relationship between age and mortality (within 72 hours) was so clear that statistical analyses were not required. The percentage mortality (in parentheses) for each group was: 50 days (10%), 70 days (15%), 90 days (60%), 110 days (90%), and 130 days (95%). The results of part 2 of this experiment indicated that the special diet was essential for survival. Of the 12 animals that were given access to only rat chow (on the floor) and water, none survived for more than 10 days (or were killed for humane reasons). On the other hand, all of the rats that were given the milk and bread feeding procedure survived. These animals returned to hopper free feeding within 7 to 14 days after the seizure.

EXPERIMENT IV: PHARMACOLOGICAL TREATMENT AND SURVIVAL

Considering the high death rate (within 48 hr) among older animals following the onset of the seizure, we decided to determine if pharmacological treatments might decrease mortality. Previous studies (15) had shown that the injection of either 10 mg/kg of diazepam or 10 mg/kg of the muscarinic antagonist scopolamine 30 min before 380 mg/kg of pilocarpine prevented the seizure as well as the usual sequelae of both brain damage and correlative decrease in glutamate decarboxylase. Similarly, Honchar *et al.* (6) reported that antecedent injection of atropine prevented lithium-pilocarpine seizures.

However we were more interested in the potential clinical application, when intervention occurs after the seizures have been initiated. Consequently, we decided to inject potentially seizure-attenuating drugs after the trigger (pilocarpine) had been administered. Atypical drugs were used as well. The possibility of pain-related factors prompted the use of morphine. Because the entorhinal cortex (which was damaged in these animals as noted in Experiment VIII) is the homologue of the parahippocampal gyrus (13) and the possibility of a panic-like factor in the animal's death was considered a relevant humane issue, alprazolam (XANAX)

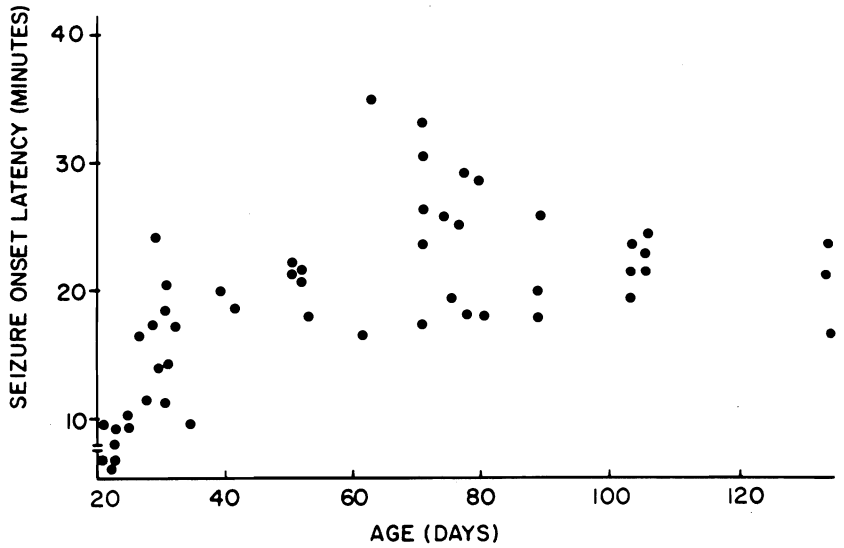

FIG. 2. Onset (in minutes) of the facial component of the lithium/pilocarpine-induced limbic seizure as a function of age (in days) at the time of the injections.

was also employed. Because one of the treatments (acepromazine) reduced mortality to less than 5% among older animals yet this observation had not been observed in a pilot study (estimated time between seizure onset and drug treatment was about 5 hours) part B of this experiment was designed if the duration of the seizure before administration of the "anticonvulsant" treatment was responsible for the discrepancy.

Subjects and Method

In part A of this experiment 56, 110-day-old males were randomly assigned to one of eight groups. All animals were seizured with 3 mEq/kg of lithium followed 4 hours later by 30 mg/kg of pilocarpine. One hour after the pilocarpine injection (and about 30 min of Phase A/B seizuring) the rats were injected subcutaneously with either: 250 mg/kg of Depakene (valproic acid), 250 mg/kg of Zarontin (ethosuxamide), 250 mg/kg of Tegretol (carbamazepine), 25 mg/kg of phenobarbital, 15 mg/kg of morphine sulfate, 1 mg/kg of Xanax, 10 mg/kg of Valium (diazepam), or 30 mg/kg of Atravet (acepromazine). Dosages were selected on the basis of availability from our pharmacy as well as feasible solubilities. The criterion for survival was 3 days since previous experiments indicated that if they lived that long they would survive, given appropriate dietary options.

In part B of this experiment 32, 120-day-old male rats were randomly assigned to four groups. After receiving the pilocarpine they were injected with 30 mg/kg of acepromazine either 30 sec, 1 hour, 2 hours or 6 hours later. Another 20 male rats, between 120 and 130 days of age, were administered the pilocarpine and then either 15 mg/kg (n=10) or 30 mg/kg of acepromazine (n=10) one hour later.

Results and Discussion

With the exception of Zarontin, diazepam and Atravet, none of the dosages administered remarkably attenuated mortality; the numbers of survivors for the these groups 3 days later ranged from 0 to 1. Whereas 3 to 4 rats that had received ethosuxamide or diazepam survived, all of the animals that had received the acepromazine survived. In part B[1] of the experiment all 10 of the rats that had received the 30 mg/kg of acepromazine survived while 6 of the 10 rats that had received the 15 mg/kg dosage survived [chi-square(1)=3.61, $0.10<p<0.05$]. These results indicate that acepromazine in combination with the postseizure milk-bread diet can maintain the animals longevity so that behavioral tests can be administered and the time course of lesion development could be studied.

EXPERIMENT V: AGE AND SEIZURE LATENCY

The ontogenetic aspects of the seizure were considered in this study. Although the characteristics of the seizure appear similar between weaning and adulthood, the latency was noted to vary. This study was designed to determine the effects of age on the onset of time for the main (Phase B) seizure.

Subjects and Method

A total of 52 male rats between the age of 20 and 140 days of age were selected as subjects from the colony room pool. All animals had been maintained in groups of 2 to 3 rats and had been bred and reared in the facility. Each animal was injected subcutaneously with 3 mEq/kg of lithium and 24 hours later with 30 mg/kg of pilocarpine. Observation procedures and criteria were the same as those noted previously.

Results and Discussion

The results are shown in Fig. 2. Seizure onset time displayed a clear inflection around 45 to 50 days of age. By 60 days of age the onset latency did not differ significantly from the values noted for the other experiments. Consequently one can conclude that the seizure onset varies from 5 min to 23 min between 21 and 55 days of age, after which it asymptoted.

EXPERIMENT V: SEX DIFFERENCES IN SEIZURE PROBABILITY AND LATENCY

One interesting observation throughout these series of studies involved gender differences. Female rats were often

inconsistent in their seizure probability. In some studies less than 10% of the females would seizure while all of their age-paired male litter mates seizured. In part A of this experiment age was investigated as a potential factor that might explain the sex differences. Part B was designed in response to the results of Part A to determine the degree to which oestrus could contribute to the probability of seizure occurrence.

Subjects and Method

In part A 60-, 90-, or 300-day-old female rats were selected as subjects; there were 20 subjects per group. Each rat was given the usual 3 mEq/kg of lithium and (24 hours later) 30 mg/kg of pilocarpine. Occurrence of a Phase B seizure was noted within the next hour. In part B of this experiment vaginal smears of 18 additional 60- to 90-day-old females were taken. They were fixed, air-dried and stained with Wright solution. Oestrus phase was determined by microscope at 100× according to the criteria of Adler (1).

Results

The percentage of female rats that seizured was 45% for the 60- to 90-day-old group and 100% for the 300-day-old group [chi square(2)=20.88, $p \leq 0.001$]. Seizure latency for the 9 females that were 90-day-old (that seizured) was 40.0±13.1 min compared to the 25.1±13.1 min average for their male (n=18) litter (4 litters) mates [previous experiments, $F(1,25)=7.96$, $p<0.01$]. For part B of this experiment females were allocated to either the estrus, metestrus, diestrus and proestrus groups. Because there was only one animal in the metestrus group it was allocated to the estrus condition. One-way analysis of variance was performed for their seizure latencies as a function of the three groups; it was not significant statistically, $F(3,13)=0.85$, $p>0.05$.

EXPERIMENT VI:EFFECTS OF PREINJECTION DISRUPTIONS

Although the onset of the main seizure was persistent with little remarkable dispersion, we were still concerned about the possible sources for the variability that did exist in the onset latency since great care had been taken in housing, maintaining and injecting the animals. The possibility was considered that variability in the time of exposure to a novel environment (such as during the preinjection period), might influence the seizure latency. This experiment was designated to test this possibility.

Subjects and Method

A total of 50 male albino rats, 90 to 100 days of age, were randomly allocated to five groups. The rats had been housed in groups (3/cage). Rats within a cage were allocated to different groups. Twenty-four hours before the test day all animals were injected with 3 mEq/kg of lithium. On the test day the experimental rats were placed in a second (novel) room for either 15 min, 30 min, 1 hour or 2 hours before injection with pilocarpine. An electric motor produced a continuous noise (45 dB) within the room. The fifth group stayed within the home-cage until the pilocarpine injection. Seizure latency (facial) was recorded to the nearest second.

Results and Discussion

The means and standard deviations for the time (in minutes) between the pilocarpine injection and the facial seizure for each of the five grous were: control (24.6±6.0), 15 min (14.1±4.1), 30 min (16.5±6.6), 1 hour (17.4±4.0) and 2 hours (18.4±5.2). A one-way analysis of variance indicated that the differences were statistically significant, $F(4,45)=2.75$, $p<0.05$. A posteriori (Scheffe's) tests indicated that the primary source of difference was between the control group and the 15 min group. Polynomial analysis indicated that the relationship between time of exposure to a novel environment before the injection of pilocarpine and the seizure latency was quadratic, $F(1,44)=5.86$, $p<0.01$, suggesting that some recovery to control levels was in progress after 15 min of exposure to the novel environment. These results strongly suggest that environmental perturbation within 15 min before the seizure-producing injection can influence its onset. The magnitude of the change in the seizure latency is within the range that might be expected by brief exposures to stress-induced endogenous glucocorticoids (12).

EXPERIMENT VII: POTENTIAL GENETIC VARIANCE

Although the onset latency of the limbic seizure was significantly but only slightly affected by preinjection manipulations we considered the possibility that genetic factors and the breeding procedures for albino rats may have influenced their seizure thresholds. Consequently we designed a study to discern if introduction of genetic pool from a wild stock of *Rattus norvegicus* might influence the latency of seizure onset. The rationale of this experiment was coupled to observations of genetic factor in human temporal lobe epilepsy (2).

Subjects and Procedure

A total of 21 Wistar albino male rats bred within the colony and 17 hybrid (brown coat) male rats also bred within the colony room were used as subjects. They were approximately 150 days of age at the time of testing. The hybrid rats were first generation offsprings of wild male rats (that had been live-trapped from a local garbage dump) and female albino rats. Albino mothers were selected instead of wild females in order to attenuate the role of early experiential factors. One wild male and an albino female were placed together until pregnancy was obvious (about 15 to 25 days). The females were then removed and housed singly before delivery. A total of 6 females (each had been housed with a different male) delivered 8–12 pups; they were culled to 8 pups per litter within 24 hours of birth.

Another 6 litters of albino pups that were born within the same week as the hybrids were also culled to 8 pups per litter. At 21 days of age the rats were placed in 3 per cage in either all hybrid, albino or mixed combinations. They were housed in this manner and maintained in normal colony conditions until the day of the seizure except during behavioral assessment between the ages of 21 and 30 days. On the day of this experiment the rats were injected subcutaneously with 3 mEq/kg of lithium and then 4 hours later with 30 mg/kg of pilocarpine.

Rats (two per session) were observed for one hour after the injection of the pilocarpine. The occurrence of either: a) body jerks, b) paddle motion with the paws or c) Phase B seizures were recorded by pressing one of several buttons that were connected to a microcomputer. The time bin for the program was set at 30 sec such that either a 0 or a 1 was recorded for each bin regardless of the number of responses. The persistence of these responses was measured by the number of bins that contained an event for the 5 consecutive

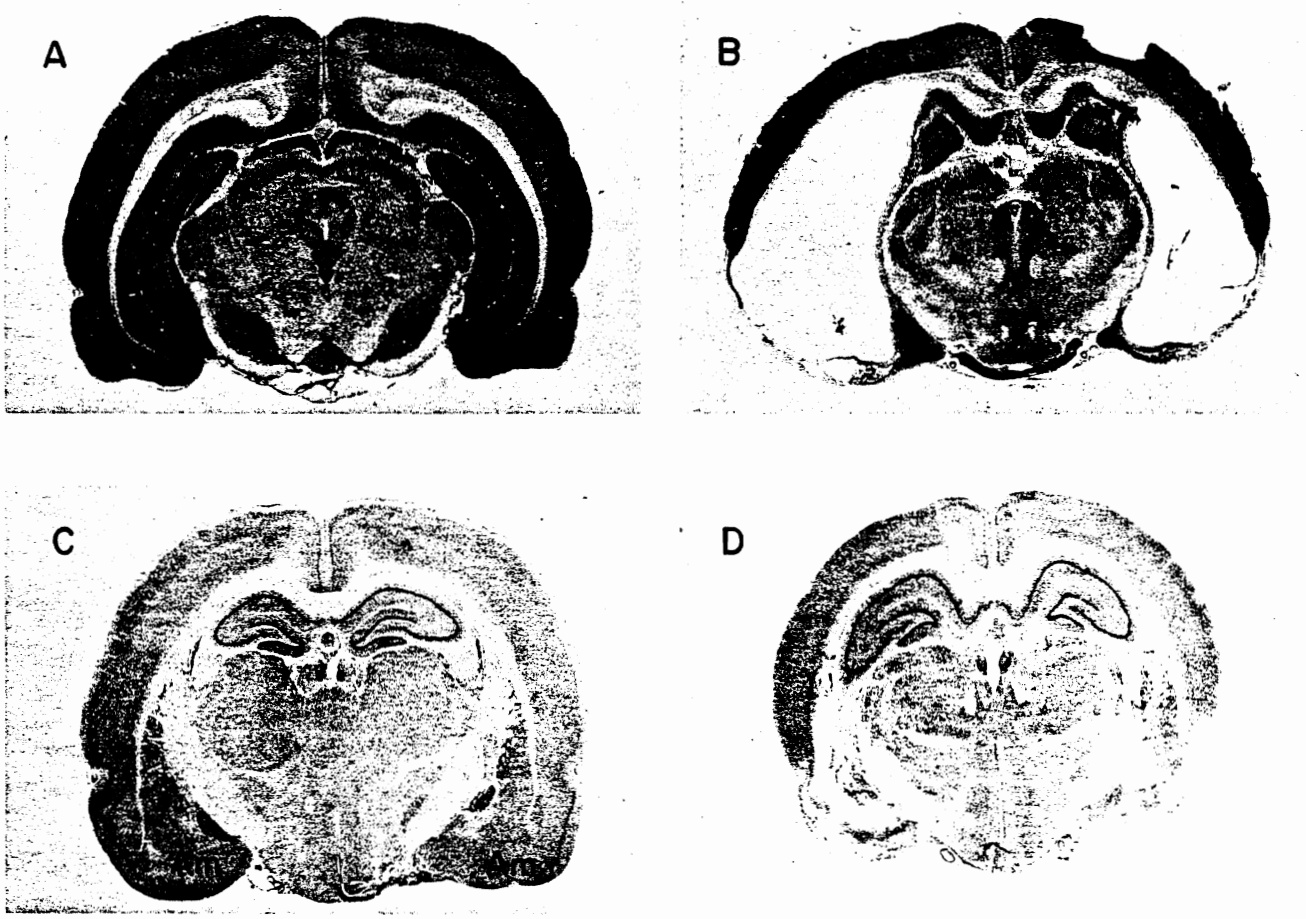

FIG. 3. Micrographs of coronal sections of sample rat brains at the level of the midbrain (A and B) and the diencephalon (C and D). (A) Brain of control rat that was injected with lithium only. (B) Brain of treated rat 5 months after one single peripheral (subcutaneous) injection of lithium and pilocarpine. Note the complete absence of the hippocampus (H) and the entorhinal/pyriform cortex (E). (C) Brain of another control animal that received lithium only. (D) Brain of another treated rat that had received the single systemic injection of lithium and pilocarpine. Note the extensive neuronal loss bilaterally within the areas of the amygdala (Am) and the entorhinal cortex (E). Except for the enlarged ventricles, the rest of the brain appeared normal. This rat was killed 3 weeks after the injection.

bins after the onset (occurrence for at least two consecutive bins) of each of the three behaviors. These measures were called frequency of jerks, paddle motions and seizures.

Results and Discussion

One-way analysis of variance indicated that there were no significant ($p>0.05$) differences between hybrid and albino rats for the three absolute measures. The means and standard deviations for the onset of the three behaviors for the albino and hybrid rats (respectively) were: paddle motions: 32.7±13.0, 36.5±7.9; seizure: 33.9±12.6, 36.6±8.2 and body jerks: 35.8±12.8, 39.5±8.9. All F values were less than 1.0. There was no significant difference between strains with respect to the persistence of the behavior once they started during the next 3 min (6 bins) for the numbers of either jerks or seizures. However the hybrid rats demonstrated significantly more, $F(1,36)=5.25$, $p=0.05$, persistent paddle movement motions (3.6±1.6) compared to the albino rats (2.4±1.3).

EXPERIMENT VIII: HISTOPATHOLOGY

The survival of rats that had displayed limbic seizures allowed investigations of the extent of brain damage. In this experiment, we briefly discuss the course and type of brain damage that was observed. The purpose of this study was to determine the feasibility of applying this model to understanding the behavioral changes that develop with the cumulative damage from temporal lobe epilepsy (9).

Subjects and Procedure

Male albino Wistar rats (approximately 100 days of age) that had been seizured either 24 hours (n=4), 1 week (n=4), 3 weeks (n=4), 50 days (n=4), or 100 days (n=7) before were killed by decapitation. Their brains were quickly removed and fixed in ethanol-formalin-acetic acid (8). After paraffin embedding the brains were sectioned (coronally) at 10 μm; every 10th section was saved and stained in thionin or toluidine blue O (pH adjusted). All the major brain structures from each of the sections for each rat brain were exam-

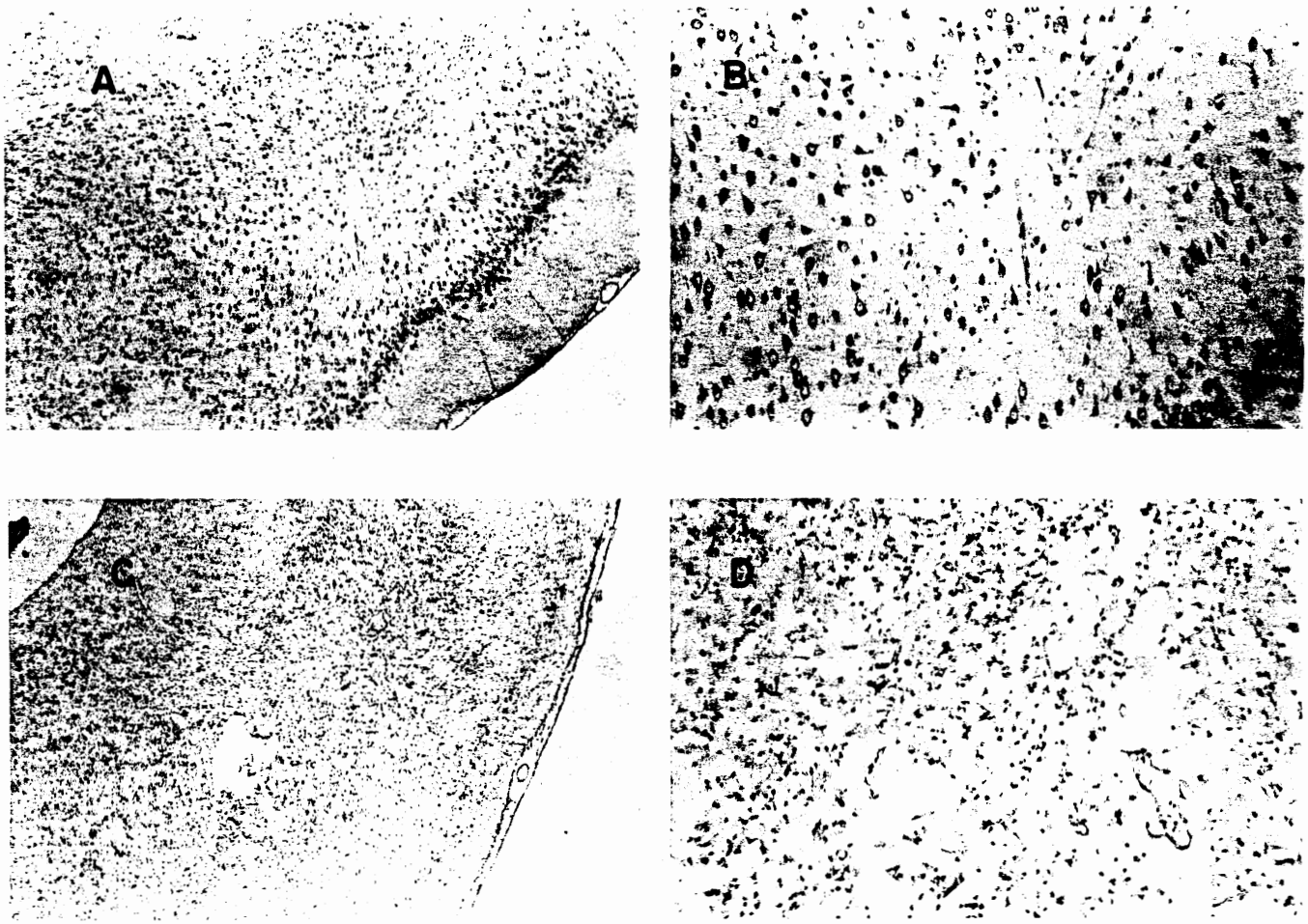

FIG. 4. Photomicrographs of the entorhinal cortex and subadjacent amygdala. (A) Section from a rat that had received lithium only (control); 30 ×. (B) Section from the same rat (A) at 100×. (C) Section from a rat that had received one subcutaneous injection of 3 mEq/kg of lithium chloride followed 24 hours later by one subcutaneous injection of 30 mg/kg of pilocarpine and killed 4 weeks later; 30×. (D) Seventy-five times magnified section from (C) of treated rat. Note the absence of neurons, the presence of glial-like nuclei and the complete tissue loss in some areas of the parenchyma.

ined between 40× and 400× between the levels of the interpeduncular nucleus (mesencephalon) and the rostral caudate-putamen (telencephalon). The cerebellums and brain stems were not examined in this study.

Results and Discussion

Three major types of lesions were evident. The first involved total (or near total) neuronal loss with replacement by reticular networks dominated by glial-like cells (Fig. 4) or by a cystic lesion (Fig. 3). The second type of lesion involved obvious neuronal cell loss and/ or increased density of glial cells that was more or less evenly distributed within the structure. The third type of lesion (Fig. 5) involved extremely focal damage that was characterized by dense staining; the constituents appeared to be combinations of tissue fragments and crystallized material (even at 1000×, with oil immersion).

The first appearance of cellular damage was clearly evident within 24 hours after the seizure. The histomorphology was dominated by neuronal loss (drop out), dense accumulation of stain within the neuronal cytoplasm and pyknotic nuclei. The damage was primarily within the entorhinal and pyriform cortices and secondarily within the ventral hippocampus and amygdala. Thalamic effects were scattered and less consistent. No other apparent pathology was evident in any of the other major structures. The histopathology was similar (if not identical) to that reported by Honchar, Olney and Sherman (6) and Turski *et al.* (14,15), who injected 400 mg/kg of pilocarpine (IP) only. Our only additional observation was the differential neuronal deterioration in or complete loss of layers II through IV within the cerebral cortices; however this was only evident if the rat displayed behaviors (e.g., deep coma) that suggested a low probability of survival within 3 days.

The extent of the brain damage after 50 or 100 days can be seen in Figs. 3 through 5. The entorhinal and pyriform cortices were decimated (Figs. 3 and 4); the extent of the lesion ranged from the most caudal pole of these structures to their rostral extent with frequent (but not always) involvement of the granular and agranular insular cortex. This type of damage was evident within one week after the seizure and did not

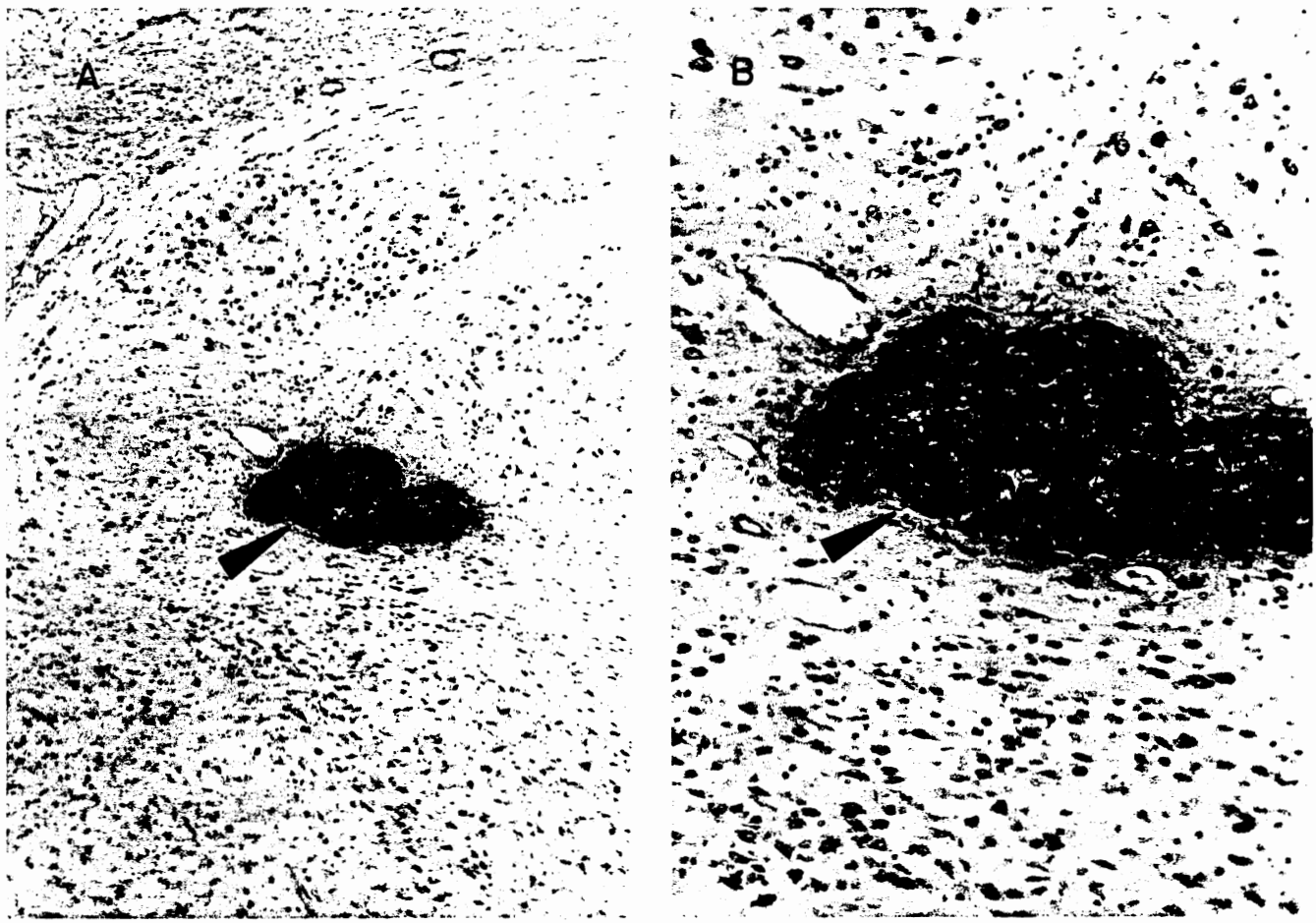

FIG. 5. An example of a focal necrotic (indicated by the wedge) area within the brain of a rat approximately 3 months after the single peripheral injection of lithium and pilocarpine. This lesion is located within the suprageniculate nucleus within the thalamus. Usually these lesions were bilateral and began to appear clearly within two weeks of the seizure. (A) 30×, (B) 75×.

change appreciably although the spatial extent of the lesion often increased up to 3 weeks after seizure (i.e., the type of damage in seizure +3 weeks and seizure +100 days did not differ). Similar damage to the adjacent amygdaloid nuclei, particularly the corticomedial group, was noted. Effectively all of the rats that survived 50 to 100 days after the seizure were partially or totally amygdalectomized.

Some but not all animals showed severe damage to the hippocampus as noted in Fig. 3B. The most systematic damage (or complete absence of tissue) occurred within the CA1 (primarily) and CA2 subsections; CA4 and the adjacent dentate gyrus were intact. In these animals the ventral subiculum was decimated and there was no evidence of any amygdaloid nuclei. Yet despite this damage the adjacent caudate-putamen and neocortex were intact; the damage ended abruptly at the structural boundaries. The lateral septum was often missing in these animals. Behaviorally, as might be expected, the animals were aggressive.

Perhaps the most intriguing types of pathology were the focal lesions noted within the thalamus (Fig. 5); such lesions were unique to this region. Although there were individual differences, there were consistent patterns. All animals who were killed 50 or 100 days after the seizure showed these lesions (usually bilaterally) in the lateral posterior nuclei, lateral dorsal nuclei, superior thalamic radiations, suprageniculate region (dorsal to the medial geniculate), ventroposterior complex and the centromedian-centrolateral group; in a few animals conspicuous lesions were noted in the gustatory nucleus, reuniens nucleus and gelatinous nucleus. About half the numbers of animals displayed similar lesions in other regions of the dorsal thalamus; there was little damage in the ventral thalamus. These lesions were not apparent within one week of the seizure but were evident within 3 weeks.

Some interesting focal aggregations of glial cells were noted in a thin section of the substantia nigra (immediately adjacent to the cerebral penduncles) and in a portion of the rostral caudate-putamen that was immediately adjacent to the internal capsule. Despite the local nature (confined within an area of about 100 μm width by 250 μm length), the cellular aggregation was apparent. However these changes were noted in only some animals. The probability is high that the abnormal cell aggregates existed in all of the animals (killed 50 days or later after the seizure) but that because of our section sampling procedure they were missed.

GENERAL DISCUSSION

The results of several studies involving more than 500 rats indicate that severe limbic motor seizures can be evoked by a single peripheral injection of lithium (3 mEq/kg) if it is followed during an optimal interval by 30 mg/kg of pilocarpine; they did not occur systematically when the injection interval was less than 2 hours and greater than 48 hours. The onset latency for the major component of the seizure (forelimb clonus, tonic head contractions and rearing) was about 30 min. The time was not significantly different (considering the standard deviations) from that reported by Honchar, Olney and Sherman who used the same dosages (6) and by Turski *et al.* (14) who used 400 mg/kg of pilocarpine only. All of the behavioral observations were similar to those reported by the above authors.

The seizure did not occur when the pilocarpine was injected first and the lithium was injected afterwards. This necessary temporal order of lithium-pilocarpine injection suggests that lithium might influence muscarinic receptors such that consequent cholinergic activity is exaggerated to levels equivalent to those produced by 400 mg/kg of pilocarpine (IP). This statement is supported by the marked similarity between the behavioral evolution of the seizure produced by either 3 mEq/kg of lithium-30 mg/kg of pilocarpine or the 380 mg/kg to 400 mg/kg of pilocarpine (14,15) and the marked correspondence in resultant brain damage. The types of initial neuronal necrosis found within the hippocampus, entorhinal-pyriform cortices, amygdala and thalamus that were reported by Turski *et al.* (14,15) were noted as extensive devastation and cystic lesions in rats that were killed 50 to 100 days after the seizure in our study.

The specificity of the lithium-pilocarpine combination is indicated by the results of our dose-dependence study; 1 mEq/kg of lithium followed by either 10, 30 or 50 mg/kg did not evoke the seizure. In addition, 12 pairings of 1.5 mEq/kg of lithium followed by 15 mg/kg of pilocarpine also did not kindle the seizure. Assuming that a longer kindling period is not required, these results might suggest that a critical threshold of lithium concentration exists that produced some all-or-none conformational change within the neuronal membrane.

The conclusion of absence of kindling is not definitive at this time. It is possible that some kindling had occurred, but was not apparent because of our procedure and lack of correlative neuroelectrical measurements. Evidence of kindling may have been evident if on the final day we had used slightly higher dosages that would have evoked seizures in 50% of nonkindled animals, thus allowing a partial super threshold response. An operationally similar procedure by Buterbaugh *et al.* (3) demonstrated that only 20 mg/kg of pilocarpine was required to induce status epilepticus in animals that had been previously kindled by classical methods.

The extent of the brain damage and the types of behavior associated with and following our procedure indicate that the major target areas in the brain involve the entorhinal-pyriform cortices, amygdala and (variably) the hippocampus. These areas should display copious concentrations of muscarinic receptors (7). However these receptors appear to initiate rather than maintain the seizure. This conclusion is supported by the capacity for muscarinic antagonists to prevent seizures (and consequent brain damage) when injected before the precipitating stimulus (pilocarpine) but not after the seizures have begun (6, 14, 15).

The temporal evolution of the damage suggests that the excitatory-induced necrosis probably occurs first within these regions of the limbic system and then spreads to include the thalamic structures and other brain areas. The majority of these changes are not likely to be reactions to a singular episode (the day of the seizure). These animals were observed to display brief seizures for weeks after the single lithium and pilocarpine injections. In addition, we have observed that certain classes of behaviors such as extreme within cage aggression (defined as killing of cage mates or chewing of another rat's tail) emerges only about 2 to 3 weeks after the first seizure, even though they have been eating regularly for more than a week. The emergence of thalamic lesions was clearly time dependent. Whereas the amygdaloid-entorhinal damage emerged within a few days and then asymptoted, the thalamic lesions became more prominent and asymptoted in severity between 25 and 50 days after the seizure. Although this could be argued to be the consequences of transneural (deafferentiating) degeneration in response to distal brain damage, the necrosis was still insidious.

It is important to emphasize that we never noted any form of lesion within the hypothalamus; this observation is commensurate with the reports of Olney *et al.* (6) and Turski *et al.* (14), although their animals were killed only 24 hours after the seizure. This lack of change is imporant in light of the occurrence of lateral hypothalamic damage-like aphagia and adipsia that occurred in all our animals that survived (with or without pharmacological adjuncts) for more than 3 days after the seizure. The capacity for these animals to respond to a bread-milk diet and to return to hopper feeding suggests that the initial response to the seizure-induced amygdaloid destruction was a functional equivalent to lateral hypothalamic damage from which the animal recovered. The implications of this phenomenon deserves further study.

The differential seizure (and survival) characteristics of male and female rats suggests that hormonal factors may be involved with epileptogenesis. Although older (over 250 days) female rats invariably seizured in our studies, younger female rats were not consistent. In some studies most of the females would seizure while in other studies none of the same-aged females would seizure. It is unlikely that body weight was a factor since young male rats of comparable weight invariably seizured. Gender-related and age-related hormonal factors have been implicated in glucocorticoid-mediated seizure damage to the hippocampal-amygdaloid complex (12). Gender differences in the responsiveness of the blood-brain barrier to lithium and pilocarpine combinations must still be determined.

One of the most important consequences of this study is that rats can survive the limbic seizure if they are given 30 mg/kg of a acepromazine within 1 hour (our more recent data suggests within 10 minutes is optimal) after the injection of the pilocarpine and then allowed to consume a milk and bread diet during the recovery period. We found that it was important to not introduce the rat to rat chow (even dust) again until after it had begun to consume all of the milk-bread mixture on a daily basis. This pattern suggests possible involvement of conditioned taste aversion mechanisms. Indeed, rats that had been seizured 2 months before by lithium and pilocarpine (but had returned to a normal diet) displayed a marked attenuation of Conditioned Taste Aversion (16).

Even though these animals ultimately show massive brain damage in limbic and thalamic structures, they do survive. This strategy may be very useful for researchers who are interested in assessing the behavioral changes over time as

the seizure-induced brain damage progresses. The continuation of electrical seizures in rats that had received the lithium-pilocarpine injections was indicated by the intermittent generalized motor seizures (months later) and the progression of the brain damage. This model may be particularly useful for understanding the patterns of behavior that might emerge or change over time during the development of temporal lobe epilepsy.

Our most recent unpublished analysis (O. Peredery and M. A. Persinger) of the histopathology of rats that had been given acepromazine (30 mg/kg) approximately 30 min after the pilocarpine (same model) indicates additional damage that was not observed in the histomorphology of the rats (no acepromazine) reported in Experiment VIII. Although the rats that received 30 mg/kg of acepromazine 30 min after the pilocarpine also demonstrated the same spatial pattern and temporal evolution (after 25 days with a peak around 50 days after the seizure) of thalamic damage, aberrant morphology in most of the reticulata region of the substantia nigra and frank gliosis within a small area of the globus pallidus have been observed. Rats that were killed 200 days after the lithium and pilocarpine injections also have reduced amygdaloid areas and enlarged ventricles. The tissue appears quite normal and evokes the challenging possibility that functional but altered reorganization of these structures might have occurred.

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'NOTE ADDED IN PROOF

Mortality within three days for the four groups that received acepromazine either 30 sec, 1 hour, 2 hours or 6 hours after the pilocarpine was 0%, 0%, 38%, and 88%, respectively.